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Preparation and lectin binding characteristics of *N*-stearyl lactobionamide liposomes

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Abstract

In order to target liposomes containing therapeutic contents to specific cells possessing galactose receptors, we synthesized the neoglycolipid, *N*-stearyl lactobionamide (*N*-SLBA), via the lactone form of lactobionic acid. Liposomes containing 0, 7.6, 10 and 15 mol% of *N*-SLBA, respectively, were used to study the impact of liposomal surface galactose density on the lectin-binding characteristics. As a lectin, *Ricinus communis* agglutinin (RCA) was used. Aggregation of *N*-SLBA liposomes was promoted with higher concentration of RCA, indicating that the galactose moieties on *N*-SLBA liposomes are accessible to lectin binding sites. RCA binding rates of liposomes increased with liposomal *N*-SLBA contents. No binding was observed between RCA and ungalactosylated control liposomes. The extent of lectin binding was also dependent on the liposomal galactose density. Rosenthal plots quantitatively revealed that the association constant (K_a) increased in proportion to *N*-SLBA contents of liposomes. These results suggest that the rate and extent of liposomal drug delivery to a target site with galactose receptors might be controlled by adjusting the *N*-SLBA contents of liposomes.

Keywords: Liposomes; *N*-stearyl lactobionamide; Active targeting; Galactose receptors; Lectin

1. Introduction

Liposomes have been widely studied as delivery systems of chemotherapeutic agents, diagnostics or immune adjuvants (Gregoriadis and Florence, 1993; Alving, 1993). In order to deliver liposomal contents to specific cells in a well-regulated manner, it would be advantageous to control liposomal uptake via unique receptors existing in the cells.

Galactose-receptors have been found in hepatocytes (Roos et al., 1985) and several immune cells including Kupffer cells and peritoneal macrophages (Taradaira et al., 1983; Haensler and Schuber, 1988). Natural glycolipids were previously used to target liposomal contents to the cells with galactose receptors. However, the use of natural glycolipids suffers from a tedious isolation procedure, low yield and insufficient purity of the products (Orr et al., 1979). In an attempt to solve problems, several research groups have synthesized glycolipids (Williams et al., 1979; Kiwada

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and Kato, 1985). Synthetic glycolipids can provide the highly purified lipids and the precisely modulated ratio of glycosylation. Ideal synthetic glycolipids should satisfy the following criteria: (1) simple synthesis with high yield, (2) stability of the bond between a lipid and a galactose moiety, and (3) projection of galactose moieties to receptor sites when incorporated into liposomes.

With these criteria in mind, we synthesized the neoglycolipid, *N*-stearyl lactobionamide (*N*-SLBA) via lactone intermediates to obtain a high yield. An amide linkage was chosen to couple galactose to stearylamine because of its stability in vivo (Bundgaard, 1985). In this study, the availability of liposomal galactose moiety to a galactose receptor was evaluated by aggregation of liposomes in the presence of *Ricinus communis* agglutinin (RCA). RCA, a dimer type lectin, has two specific binding sites for β-D-galactose residues, and has been used as a model system of a galactose receptor (Hoekstra and Duzgunes, 1986 ; Yoshioka et al., 1993). Furthermore, we elucidated whether the galactolipid contents of liposomes could modulate the binding rate and extent of liposomes to lectin.

2. Materials and methods

2.1. Materials

L-α-Phosphatidylcholine, dimyristoyl (DMPC), cholesterol, lactobionic acid hemicalcium salt, stearylamine, α-tocopherol, *Ricinus Communis* agglutinin (RCA) and fluorescent isothiocyanate (FITC)-RCA conjugate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Amberlite IR-120 (plus) was supplied from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). Absolute methanol was purified by distillation before use. All other chemicals were of reagent grade and used without further purification.

2.2. Synthesis of N-stearyl lactobionamide (N-SLBA)

Lactobionic acid hemicalcium salt was converted to free lactobionic acid on a cation exchange column (1.5 × 20 cm, Amberlite IR-20) with 2 N NaCl and then with 2 N HCl. During the ion exchange process, the water jacket of column was circulated alternately with hot (100°C) and cool (25°C) water to promote the exchange of calcium cation of lactobionic acids with hydrogen ion of HCl. Eluted free lactobionic acids were dried in vacuum and converted to lactobiono-1,5-lactone by repeated evaporation from methanol and ethanol.

Lactobiono-1,5-lactone and stearylamine (molar ratio of 1:1, 2.8 mmol each) were dissolved in 10 ml of anhydrous methanol with gentle heating in a water bath and the reaction mixture was stirred for 12 h at room temperature. The formed precipitate was filtered, washed with cold methanol and chloroform in that order, and air dried. The repeated recrystallization from methanol gave a white crystalline power of *N*-SLBA.

N-SLBA was identified with a differential scanning calorimeter (DSC, Perkin-Elmer, Buckinghamshire, UK), a FT-IR (Perkin-Elmer, Buckinghamshire, UK) and a nuclear magnetic resonance (400 MHz ^{1}H-NMR, JEOL JEM-GSX, Tokyo, Japan). For IR spectra, compounds were prepared as KBr tablets. For NMR measurements, *N*-SLBA was dissolved in DMSO-d_6, and tetramethylsilane was used as an internal standard.

2.3. Preparation of liposomes containing N-SLBA

Liposomes composed of DMPC, cholesterol, α-tocopherol and *N*-SLBA were prepared by reverse-phase evaporation (Kim et al., 1993; Kim and Park, 1994) with minor modifications. The molar ratio of *N*-SLBA varied to prepare the liposomes composed of 0, 7.6, 10 and 15 mol% of *N*-SLBA. Briefly, 19.2 μmol of DMPC, 9.6 μmol of cholesterol and 0.24 μmol of α-tocopherol as an antioxidant were dissolved in chloroform. *N*-SLBA (2.4 μmol, 3.2 μmol and 5.2 μmol for 7.6 mol%, 10 mol% and 15 mol% *N*-SLBA liposome, respectively) dissolved in methanol was then added to the above chloroform solution. The organic phase was removed using a rotary evaporator at reduced pressure (360 mmHg) and then

the dried lipid film was redissolved in 4.2 ml of a 1:2 mixture of chloroform:isopropyl ether. A 1.4 ml sample of 0.01 M phosphate buffer (pH 7.0) was added and two phases were emulsified by the repeated process of vortexing for 1 min and incubating for 30 s in a 30°C water bath. The trace of organic phase was removed further under reduced pressure (360 mmHg) at room temperature. The resulting liposomes were allowed to be annealed at room temperature for 30 min followed by rapid cooling and then ultracentrifuged at 180000 *g* for 15 min. The pelleted *N*-SLBA liposomes were resuspended in 0.01 M phosphate buffer (pH 7.0). The resulting *N*-SLBA liposome suspension was ultrafiltrated with 0.6 μm polycarbonate filters to eliminate larger size vesicles. Concentration of DMPC in the liposomes was determined by the Stewart assay (Stewart, 1959) with minor modifications.

2.4. RCA-mediated aggregation rate of liposomes

Different amounts of RCA (55, 110, 165 and 220 μg/ml) were added to the liposome suspension composed of 15 mol% *N*-SLBA (equivalent to 0.48 μmole of DMPC) in a cuvette pretreated with 5% bovine serum albumin solution. The pretreatment with albumin reduced the non-specific binding of RCA to the cuvette. For the lectin binding rate determination, 110 μg/ml of RCA was added to liposomes composed of various percentages (0, 7.6, 10 and 15 mol%) of *N*-SLBA. The final volume of the mixture of the liposome suspension and RCA was adjusted to 3.0 ml. The aggregation rate of liposomes was estimated by time-dependent increase in turbidity as measured by absorbance at 360 nm at designated time.

2.5. Extent of N-SLBA-liposome binding to lectins

FITC-labeled RCA was used as a fluorescent probe to detect the lectin binding of *N*-SLBA liposomes. The mixtures of RCA and liposomes composed of 0, 7.6, 10.0 and 15.0 mol% of *N*-SLBA, respectively, were shaken for 2 h at 37°C, and then ultracentrifuged at 180000 *g* for 15 min. The study was executed by increasing the amounts of RCA (0 to 160 μg) in the different test tubes containing the liposome suspension equivalent to 0.48 μmole of DMPC with the final volume of 2 ml. The amounts of unbound RCA in the supernatant were determined by measuring the fluorescence intensity of FITC-labeled RCA at 490 nm excitation wavelength and 520 nm emission wavelength. The amounts of bound RCA were calculated by subtracting the amounts of unbound RCA from the total amounts of RCA.

3. Results and discussion

3.1. Synthesis and identification of N-SLBA

Stearylamine was chosen as the lipid anchoring galactose. The use of stearylamine is advantageous in that we can use the inherent amine group of stearylamine to conjugate galactose without introducing amine-containing spacer arms to lipids. To synthesize *N*-SLBA via an amide bond between the amine group of stearylamine and the carboxyl group of lactobionic acid, lactobionic acid was first converted to lactones by dehydration of solvent (Fig. 1). The conversion to lactones was a crucial step to obtain *N*-SLBA with a higher yield, since the carboxyl group of lactobionic acid lacks reactivity to amines even in the presence of catalysts such as carbodiimides (Williams et al., 1978) whereas lactones are highly reactive to amines.

The dehydrated lactobionic acids can produce two kinds of lactones, 1,4-lactone and 1,5-lactone. However, in our temperature conditions of 40°C, 1,5-lactone appeared to be the major lactone form because 1,5-lactone is preferentially formed at the temperature slightly higher than 25°C while 1,4-lactone is formed at exceedingly higher temperatures. The formation of the lactone was confirmed by the IR spectrum of the dehydrated lactobionic acid which showed a peak at 1750 cm^{-1} of a carbonyl group but no peak at 1650 cm^{-1} of a carboxyl group. It indicates that the carboxyl group of lactobionic acid was converted to a carbonyl group in lactones. The reaction between lactones and stearylamine produced a white crystalline powder of *N*-SLBA (M.W. 609) with an

yield as high as 56.8%. The amide group of *N*-SLBA was identified by a characteristic peak at 1650 cm^{-1} in the IR spectrum, and by a weak triplet at 7.5 ppm in the NMR spectrum (Kim et al., 1992). Amide bonds have been known to be relatively stable in vivo (Bundgaard, 1985). Thus, *N*-SLBA liposomes where galactose moieties are linked via amide bonds may achieve more effective delivery in vivo by retaining the surface galactose in blood stream, and exposing more amount of galactose to a target site.

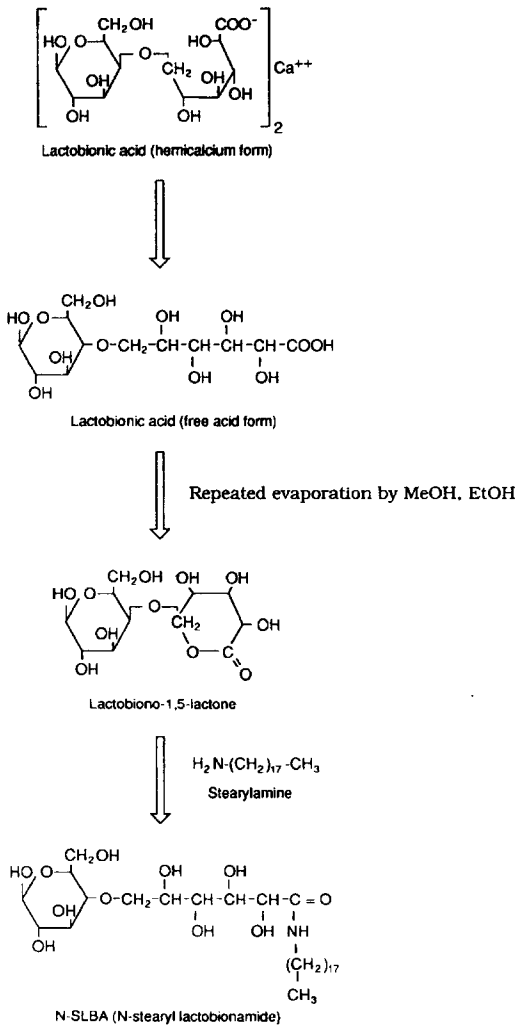

Fig. 1. Reaction scheme for the synthesis of *N*-stearyl lactobionamide.

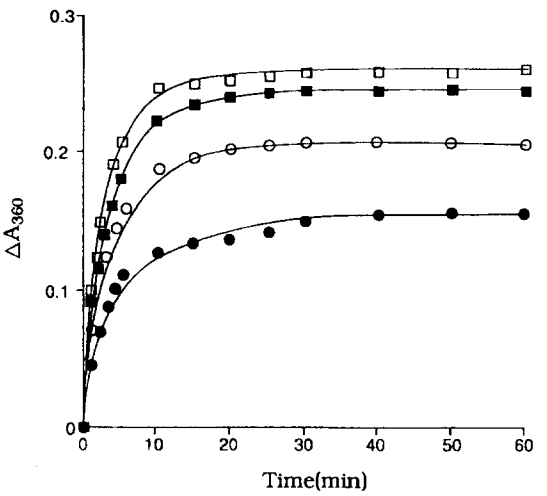

Fig. 2. Time course of turbidity changes due to the aggregation of neogalactosylated liposomes after addition of various amounts of *Ricinus communis* agglutinin to liposomes composed of 15 mol% *N*-SLBA. Key : ●, 55 μg/ml RCA; ○, 110 μg/ml RCA; ■, 165 μg/ml RCA; □, 220 μg/ml RCA.

3.2. Accessibility of galactose moieties on N-SLBA liposomes to RCA

If *N*-SLBA liposomes are to target galactose receptors, galactose moieties in *N*-SLBA liposomes should project toward aqueous environments so as to be accessible to the receptors. Given that the polar amine group of stearylamine in liposomes is considered to project toward the water phase, we hypothesized that the polar galactose which replaced the amine group of stearylamine in *N*-SLBA liposomes may orient to aqueous surroundings well enough to be recognized by a galactose receptor. The hypothesis was evaluated by studying the binding of *N*-SLBA liposomes to RCA. Once *N*-SLBA liposomes bind to RCA, the lectin will promote liposomal aggregation by cross-linking vesicles, which increase the turbidity of the liposomal suspension. When higher amounts of RCA were mixed with liposomes containing 15 mol% of *N*-SLBA, more liposomes were aggregated (Fig. 2). It supports our hypothesis that *N*-SLBA liposomes have surface galactose moieties accessible to a galactose recognizing system. In addition, the extent of aggregation reached an asymptomatic level above

165 μg/ml of RCA, indicating a saturation of binding sites (Fig. 3).

3.3. Effect of surface sugar density on the lectin binding kinetics of N-SLBA liposomes

To test whether we can control the delivery rate of liposomes by liposomal surface sugar density, we studied the effect of liposomal surface sugar density on the binding kinetics of liposomes to RCA. The lectin binding rate of *N*-SLBA liposomes was determined by the measurement of aggregation rate of liposome. No aggregation was observed in the liposomes without *N*-SLBA. However, as the content of *N*-SLBA increases from 7.6 to 10 and 15 mol%, the initial agglutination rate of liposomes increased proportionally and reached a plateau level within 30 min (Fig. 4), when drawn with the difference of absorbance from the initial absorbance (indicated as ΔA, which means A_t-A_0, where A_0 is the absorbance at time zero) versus time in min. The kinetic parameters of binding were obtained by plotting the logarithmic value of $(A_\infty - A_t)/A_\infty$ versus time as shown in Fig. 5, where A_∞ is the absorbance at infinity and A_t is the absorbance at time 't'. Since the plateau level was reached within 30 min, we assumed absorbance at 2 h as A_∞. The plot shows that the binding of *N*-SLBA liposomes to RCA undergoes a relatively slow initial phase and faster later phase. Overall this process appears to follow pseudo-first order kinetics. Our results are in agreement with previous finding of Orr et al. (1979) which reported the pseudo-first order kinetics of lectin-mediated aggregation in the liposomes containing galactosylated cholesterol. Table 1 shows that initial binding rate constants of liposomes to RCA are dependent on the *N*-

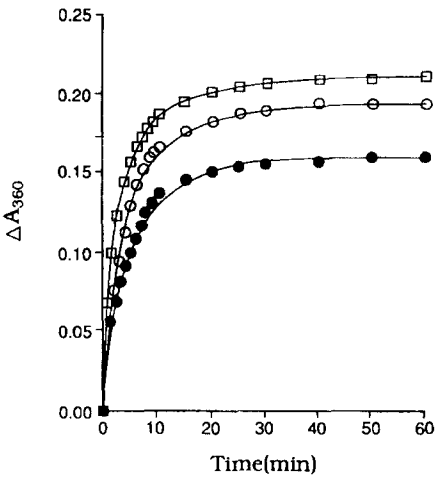

Fig. 4. Influence of surface galactose density on the lectin binding rate of the neogalactosylated liposomes. *Ricinus communis* agglutinin (110 μg/ml) was added to liposomes composed of various percentages (0–15 mol%) of *N*-SLBA. Key : ●, 7.6 mol%; ○, 10 mol%; □, 15 mol%.

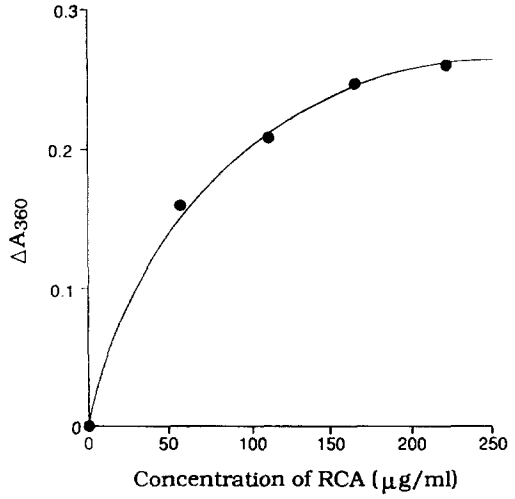

Fig. 3. The extent of aggregation at 2 h incubation as a function of *Ricinus communis* agglutinin concentration.

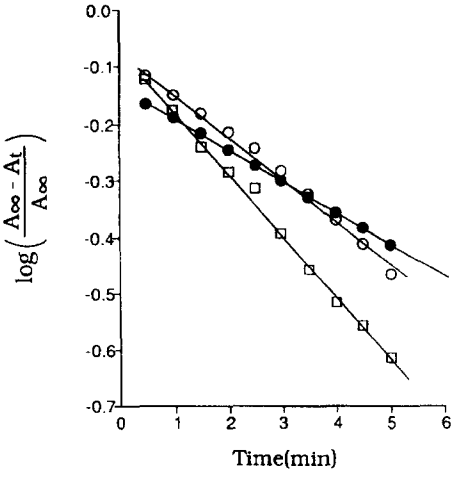

Fig. 5. Pseudo-first order kinetic plot of aggregation. Key : ●, 7.6 mol%; ○, 10 mol%; □, 15 mol%.

Table 1
Initial agglutination rate constants of *N*-SLBA liposomes to RCA

Mole fraction of *N*-SLBA (mol %)	Apparent rate constant (min^{-1})
7.6	0.130
10.0	0.176
15.0	0.254

SLBA contents. It indicates that the liposomal surface sugar density mainly controls the binding kinetics of liposomes to a galactose-recognizing system.

3.4. Effect of liposomal surface galactose density on the extent of binding to RCA

If surface galactose density affects the extent of liposomal binding to RCA, we may control liposomal binding to galactose receptors by adjusting *N*-SLBA contents. In this regard, we determined the bound amounts of RCA at equilibrium in the presence of liposomes with *N*-SLBA contents of 7.6, 10 and 15 mol%. Liposomes without *N*-SLBA were compared as a control. The extent of binding at equilibrium was assumed as the amount of RCA bound to liposomes at 2 h incubation, based on the liposome binding rates shown in Fig. 4. As liposomal galactose contents increased, more amount of RCA bound to liposomes over the whole range 0–160 μg of RCA (Fig. 6). In contrast, the liposomes without *N*-SLBA did not show any binding.

Rosenthal plots were applied to quantitatively analyze the binding of *N*-SLBA liposomes to RCA (Rosenthal, 1967). The binding data fit well the following equation:

$$[B]/[F] = K_a n[Lt] - K_a[B]$$

where [B] and [F] are molar concentrations of bound and free RCA, respectively, 'n' is the number of binding sites, n[Lt] is the binding capacity of liposomes, and K_a is the association constant. When [B]/[F] was plotted against [B], all the liposomes with 7.6, 10 or 15 mol% of *N*-SLBA showed linear plots, suggesting that only one class

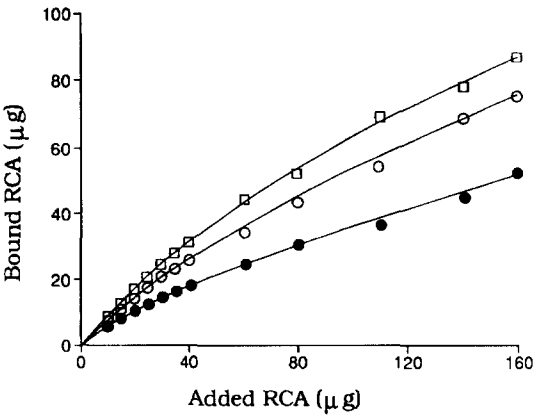

Fig. 6. Influence of surface galactose density on the lectin binding extent of the neogalactosylated liposomes. Various amounts of *Ricinus communis* agglutinin (0–160 μg/ml) were added to liposomes composed of various percentages (7.6, 10 and 15 mol%) of *N*-SLBA. Key : ●, 7.6 mol%; ○, 10 mol%; □, 15 mol%.

of binding sites exists (Fig. 7). However, the three different slopes reveal that the liposomal galactose contents strongly affect K_a values. Table 2 shows that association constant and binding capacity of *N*-SLBA liposomes increase with *N*-SLBA contents due to increase in galactose moieties on the surface of *N*-SLBA liposome. The K_a of 15 mol% *N*-SLBA liposomes is almost 3.8 fold higher than the K_a of 7.6 mol% *N*-SLBA liposomes. It pro-

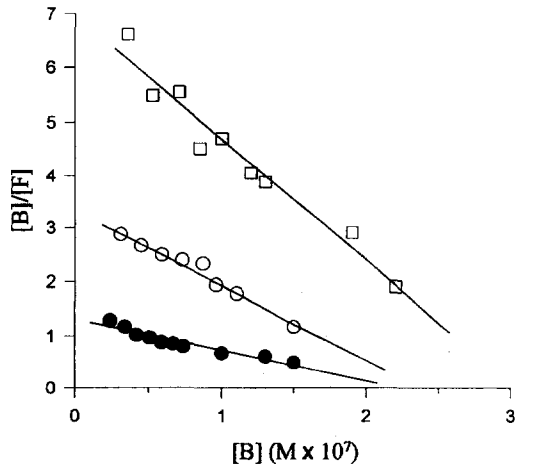

Fig. 7. Rosenthal plots for characterizing the binding of the neogalactosylated liposomes to *Ricinus communis* agglutinin. Key : ●, 7.6 mol%; ○, 10 mol%; □, 15 mol%.

Table 2
Association parameters of *N*-SLBA liposomes to RCA at 37°C

Mole fraction of *N*-SLBA (mol %)	Association constant (M^{-1})	Binding capacity (M)
7.6	0.58×10^7	2.26×10^{-7}
10.0	0.68×10^7	2.38×10^{-7}
15.0	2.27×10^7	3.06×10^{-7}

vides evidence that the liposomal surface galactose density plays an important role in determining the extent of liposomal binding with lectins.

In conclusion, these results suggest that the synthetic galactolipid, *N*-SLBA, can be incorporated successfully to liposomes, and that the rate and extent of liposomal binding to lectins are highly dependent on the surface sugar density. Furthermore, it implies that the rate of targeting as well as the extent of delivery to the hepatocytes and several immune cells including Kupffer cells and peritoneal macrophages might be controlled by adjusting the surface galactose density of liposomes.

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